PATENT SPECIFICATION

1,107,790

NO DRAWINGS

1,107,790

Date of Application and filing Complete Specification: 23 March, 1967.
No. 13626/67.
Application made in Germany (No. B86402 IXb/42 l) on 29 March, 1966.
Application made in Germany (No. B88815 IXb/42 l) on 8 Sept., 1966.
Application made in Germany (No. B89784 IXb/42 l) on 11 Nov., 1966.
Complete Specification Published: 27 March, 1968.

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ERRATA

SPECIFICATION No. 1,107,790

Page 1, line 9, *for* "seriological" *read* "serological"
Page 1, line 36, *for* "the" *read* "these"
Page 2, line 5, *for* "seevral" *read* "several"
Page 2, line 29, *for* "comprises" *read* "comprise"
Page 3, line 55, *for* "pnuemonia" *read* "pneumonia"
Page 3, line 60, *for* "casual" *read* "causal"
Page 4, line 2, *for* "properation" *read* "preparation"
Page 4, line 6, *for* "sigificant" *read* "significant"
Page 6, line 27, *for* "2 thienyl" *read* "2-thienyl"
Page 7, line 41, *for* "Microorganisms" *read* "Microorganism"

THE PATENT OFFICE

ERRATA

SPECIFICATION No. 1,107,790

Amendment No. 2

Page 1, line 12, *for* "metabolical" *read* "metabolic"
Page 3, line 28, *for* "could also" *read* "also could"
Page 4, line 32, *for* "accelator" *read* "accelerate"
Page 6, line 9, *for* "recrsytallised" *read* "recrystallised"
Page 6, line 21, *after* "methyl-" *delete* "5-"
Page 7, line 20, *for* "(Tween 20" *read* "("Tween 20")"
Page 7, line 38, *after* "upon" *insert* "the"
Pages 9 and 10, column 3 and 5, *for* "colouration" *read* "coloration"

THE PATENT OFFICE
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Index at acceptance:—C6 F1B; C2 C(2B27, 2B30, 2B31, 2B33, 2B34)
Int. Cl.:—C 12 k 1/06

COMPLETE SPECIFICATION

A process and Diagnostic Agents for the Detection of Bacteria

We, C. F. BOEHRINGER & SOEHNE G.M.B.H., of Mannheim-Waldhof, Germany, a Corporation organised under the laws of Germany, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention is concerned with a process and diagnostic agents for the detection of bacteria.

For the rapid and effective combatting of infectious diseases, it is desirable to be able to detect as quickly as possible the microorganisms causing the disease and to be able to differentiate them with certainty. Besides the classical seriological methods, there are known a number of investigational processes in which the enzymes formed by the microorganisms are detected with the help of a colour indicator or in which the metabolical products of specific bacteriophages are utilised for the detection of the bacteria.

Thus, for example, the detection of some kinds of bacteria is possible by the Griess nitrite test in which nitrate is reduced to nitrite by the nitrite reductase formed by the bacteria, the presence of nitrite then being detected with sulphanilic acid and α-naphthylamine (c.f. H. J. Walther, Ärztl. Lab., *6*, 287/1960). Since this is only a non-specific and not very reliable detection of nitrite and does not constitute a specific detection of bacteria, it has only a limited and conditional importance (c.f. Linzenmeier et al., Klin. Wschr., *41*, 919/1963).

Another known method for the detection of bacteria depends upon the reduction of the colourless 2,3,5-triphenyl-tetrazolium chloride to the red-brown coloured triphenyl-formazane by reductases peculiar to bacteria. This reaction is completely non-specific and, therefore, does not permit a differentiation of the bacteria present (c.f. Simmons et al., Lancet, *1*, 1377/1962).

From U.S. Patent Specification No. 3,122,480, there is known a method for the detection of strains of *Pseudomonas* which depends upon the reaction of enzymes peculiar to bacteria with p-phenylene-diamine and substituted naphthol derivatives to give coloured compounds (indophenol blue). For the carrying out of this detection method, the microorganisms to be identified must first be caused to grow by the use of selective nutrient media, incubation periods of about 18 hours at 37°C. thereby being necessary. For the isolation of an important cause of infections of the urinary tract, namely *Pseudomonas aeruginosa,* special nutrient media are required, thereby making the use of this known process even more difficult. However, the decisive disadvantage of this process is the fact that *Escherichia coli* and the enterococci, such as *Streptococcus faecalis,* are not detected, although the two are found to be the cause in more than two thirds of all non-specific infections of the urinary tract.

For the detection of a special strain of bacteria, namely the gonococci, in U.S. Patent Specification No. 2,970,945 there has been described the use of tetramethyl-*p*-phenylene-diamine, the indicator substance thereby being oxidised to a dyestuff by oxidases peculiar to the bacteria. Because of the atmospheric sensitivity of the indicator

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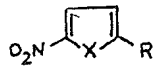
substance and of the limitation to one strain of bacteria, a general importance cannot be attributed to this reaction.

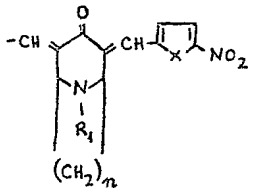
Finally, a number of publications have appeared which describe the detection of bacteria with the help of bacteriophages (c.f. H. J. Raettig, "Bakteriophagie", pub. Gustav Fischer-Verlag, Stuttgart, 1958, page 53). However, for seevral reasons, it has not been possible to develop this method into one for routine use because "there are hardly any monovalent bacteriophages for one strain of bacteria and . . . . these few monovalent phages easily become polyvalent and thus extend to other bacterial strains". Furthermore, since bacteria are very frequently phage-resistant, the already very laborious and time-consuming detection is, in addition, not dependable and is unsuitable in practice.

We have now found that compounds of the general formula: —

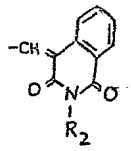


(1)

wherein X is an oxygen or sulphur atom or an alkylated nitrogen atom and R is one of the following radicals: —



(a)



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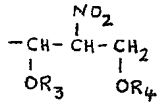

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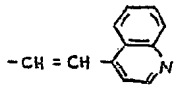

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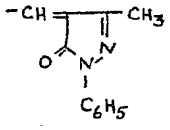


(e)

wherein R_1 is a saturated or unsaturated aliphatic, aromatic, araliphatic, acyl, hydroxy or acyloxy-aliphatic radical, R_2 is a hydrogen atom or a lower alkyl radical, R_3 and R_4 are both hydrogen atoms or together form an isopropylidene radical and n is 2 or 3, are suitable for the detection of bacteria, especially in urine, by a colour reaction.

This new type of detection for bacteria depends upon the fact that these compounds (1) are metabolised by bacteria to give dyestuffs, the reaction being, in some cases, even specific for strains of bacteria.

The process according to the present invention is characterised in that the medium to be investigated is brought together with a liquid or solid nutrient medium and a non-growth-inhibiting amount of a compound of general formula (1) and, after an incubation time of 1—18 hours at 20—37°C., the colour change is evaluated.

The diagnostic agents according to the present invention comprises a liquid or solid nutrient medium and a non-growth-inhibiting amount of a compound of general formula (1).

With the new test according to the present invention, there can be carried out a general detection for bacteria, i.e. a qualitative total determination of microorganisms.

In other words, it is possible to detect a plurality of microorganisms which can, for example, be present at the same time in an infection of the urinary tract, by means of a common colour reaction. However, there can also be achieved a differentiation of the microorganisms, i.e. a qualitative detection of individual types of microorganisms. In other words, individual types of microorganisms, for example, only *Escherichia coli*, can, on the basis of a colour reaction specific for the micro-organisms in question, be detected and exactly determined. The sample to be tested can hereby also be tested successively with several different diagnostic agents according to the present invention, those compounds of general formula (1) being expediently chosen which give easily differentiateable, specific colour reactions. In addition, preferred forms of these combinations also contain selectively effective bactericidal or bacteriostatically-effective substances and/or differentiating nutrient media which provide especially favourable growth conditions for the type of microorganism to be detected but which inhibit undesired accompanying microorganisms.

Since, in the case of some of the compounds of general formula (1), the time of the coloration depends upon the number of microorganisms originally present, by means of the process according to the present invention and with the help of the diagnostic agents according to the present invention, semi-quantitative determinations of the number of microorganisms is also possible.

For carrying out the process according to the present invention, the solution to be investigated, such as urine, milk, drinking water or the like, is added to a nutrient broth in, for example, a test tube the broth containing a sufficient but non-growth-inhibiting amount of one of the compounds of general formula (1). An upper limitation of the amount of compounds of general formula (1) to be added is necessary because the compounds (1) possess certain bactericidal or bacteriostatic properties. Because of this fact, it was, indeed, to have been expected that, in the presence of these compounds, a sufficient bacterial growth would no longer have been possible so that the compounds could also not be metabolised by bacteria to give dyestuffs. Surprisingly, however, for the detection reaction according to the present invention, there are necessary amounts which are substantially smaller than the minimum inhibiting concentrations and no disturbances occur in the carrying out of the test.

Some of the compounds of general formula (1) are only sparingly soluble. In such cases, it is advantageous to add a solubiliser in order to achieve sufficient concentrations for the colour test. The test mixture is left for a few hours at room temperature or at an incubation cabinet temperature of 37°C. and the colour changes evaluated from time to time. Instead of a liquid nutrient broth, the compounds of general formula (1) can also be mixed with a warmed, liquid nutrient agar and, after cooling, the liquid to be tested placed upon the solidified nutrient medium.

Preferred forms of the diagnostic agents according to the present invention are nutrient-containing tablets or nutrient-containing filter paper strips which already contain the compound (1) in question. In the case of the use of tablets, for the carrying out of the detection reaction these only need to be dissolved in a sample of the liquid to be tested. After an incubation time of, on an average, 1—18 hours, the colour change can be evaluated directly. In the case of the use of filter paper test strips, the strips are dipped for a short time in the solution to be tested, then placed in a small, sterilised test tube and kept for some time at room temperature or at 37°C. The result can also be read off directly. In order to keep the tablets or filter paper strips storable, they are expediently sterilised with ethylene oxide and sealed between sterilised foils which can easily be torn off before use.

The advantages of the process according to the present invention and of the diagnostic agents according to the present invention are, in the first place, the simple and sure way in which they can be handled, as well as the reliable indication of microorganisms present. In particular, the causal microorganisms which occur in the case of non-specific infections of the urinary tract, such as *Escherichia coli, Streptococcus faecalis, Proteus mirabilis, Staphylococcus aureus, Klebsiella pnuemonia* and *Pseudomonas aeruginosa,* can be dependably detected. Furthermore, according to the present invention it is also possible not only to detect the general presence of bacteria but also to carry out qualitative and semi-quantitative individual determinations of special types of microorganisms, without first having to carry out laborious, expensive and time-consuming isolations of the casual microorganisms in pure cultures.

It is also possible to carry out the detection reactions, without the use of an incubation cabinet, at room temperature but in such cases somewhat longer incubation periods must be taken into account. Whereas with the use of an incubation cabinet, times of only 1—12 hours are necessary, at room temperature it is usually necessary

to have to wait for 4—18 hours in order to obtain a clear coloration. In the case of the use of prepared nutrient media, test papers and tablets, the usual properation times are rendered unnecessary and it is sufficient to use test tubes or pipettes as aids.

We have also found that the incubation times needed in actual practice can be considerably shortened and a clearer distinction between "normal" and "asymptomatic/sigificant" ranges of the number of microorganisms can be achieved when the material to be investigated, such as a nutrient broth containing microorganisms or urine containing microorganisms and mixed with a nutrient medium, is incubated without the addition of one of the compounds of general formula (1), after which a compound of general formula (1) is added, incubation continued for a short period of time and the colour change then evaluated.

In the case of a urine investigation, a period of about 9—10 hours is necessary when a compound of general formula (1) is added immediately, whereas with the use of this modified procedure, the end result can be obtained after only about 5—6 hours.

Finally, we have also found that the incubation time required in actual practice can also be significantly reduced and a clearer distinction between "normal" and "asymptomatic/significant" ranges of the number of microorganisms can be achieved when the material to be investigated, such as a nutrient broth containing microorganisms or urine containing microorganisms and mixed with a nutrient medium, is additionally mixed with coenzymes, as well as, if desired, sulphhydryl group-containing compounds and/or enzyme-activating heavy metal ions.

As coenzymes, there can be used, for example, nicotinamide-adenine-dinucleotide (NAD) or nicotinamide-adenine-dinucleotide phosphate ((NADP) or the reduction products thereof.

As sulphhydryl group-containing compounds there are preferably used cysteine, reduced glutathione, cysteamine or β-mercapto-ethanol. Instead of the compounds with free sulphhydryl groups, there can also be used the corresponding dehydrated disulphide compounds.

Enzyme-activating heavy metal ions are preferably provided by the salts of manganese and copper.

These additives accelater the coloration not only in the case in which a compound of general formula (1) is present initially but also in the case in which a compound of general formula (1) is added after a preliminary period of incubation. Since the time factor plays a decisive part for the practical application of the process according to the present invention, the working method using a preliminary incubation and subsequent addition of a compound of general formula (1) is preferably further accelerated and improved by the addition to the nutrient medium of the above-mentioned further additives.

Good effects are even obtained by the addition of the coenzymes alone. However, optimum results are obtained when, in addition, sulphhydryl group-containing compounds and heavy metal ions, preferably in the form of manganous salts, are also present.

Thus, for example, after a preliminary incubation of only 1—2 hours at 37°C., the addition of a non-growth-inhibiting amount of a compound of general formula (1) and a subsequent incubation for 30 minutes at 37°C. permits the clear recognition of a large number of microorganisms. In the case of incubating for a further 1—2 hours, smaller numbers of microorganisms of up to 100,000 per millilitre, which are, however, also of interest in practice, can also be identified.

For this variant of the process according to the present invention, it has proved to be particularly useful to employ gelatine capsules which contain all the components of the nutrient broth and the above-mentioned additives. The contents of a capsule, which are sufficient for one urine investigation, are shaken out into the urine sample. After the preliminary incubation period, a compound of general formula (1), preferably in the form of a stable solution, is added thereto. After a relatively short further period of incubation, the coloration is evaluated.

The compounds of general formula (1) are new. They can be prepared by one of the *per se* known methods described in the following:

I. General procedure for the preparation of N-substituted 2,4-di-(5-nitro-2-furfurylidene)-granatan-3-one and 2,4-di-(5-nitro-2-furfurylidene)-tropinone derivatives of Formula 1a):

0.1 mol of the N-substituted norgranatan-3-one or -tropinone in question is heated under reflux and with stirring for 3 hours with 28.2 g. (0·2 mol) 5-nitrofurfural

in 50 ml. acetic anhydride. The reaction mixture is thereupon stirred with ice water, the precipitated crystalline substances filtered off with suction and recrystallised. The compounds set out in the following Table were prepared in this manner:

TABLE

Compound No.	R_1	X	n	yield	m.p. °C.	recrystallised from
I	$-CH_2CH_2OCOCH_3$	0	3	83%	144—145	alcohol/glacial acetic acid
II	$-CH_3$	0	3	63%	226—228	isopropanol
III	$-CH_3$	0	2	18%	203—204	isopropanol
IV	$-C_2H_5$	0	3	56%	226—227	dimethyl formamide
V	$-C_6H_5$	0	3	5%	218—220	butanol
VI	$-CH_2C_6H_5$	0	3	74%	215—217	dioxan
VII	$-CH_2CH=CH_2$	0	3	37%	186—187	isopropanol
VIII	—OH	0	3	9%	254 (decomp)	toluene
IX	$-CH_2CH_2C_6H_5$	0	3	94%	158—159	isopropanol
X	$-COCH_3$	0	3	14%	255 (decomp)	acetic acid
XI	$-CH_3$	S	3	43.3%	210—212	acetic acid

The N-hydroxy-norpseudopelletierin required as starting material for the preparation of compound (VIII) is also new and can be prepared in the following manner:

146 g. (1 mol) acetone dicarboxylic acid, 56 g. sodium acetate trihydrate, 69.6 g. hydroxylamine hydrochloride and 400 ml. 25% glutaric dialdehyde solution are dissolved in 1000 ml. water. The evolution of carbon dioxide commences immediately. The solution is left to stand for 3 days at room temperature. It is then rendered alkaline with a solution of sodium hydroxide and extracted with chloroform. The extract is dried and evaporated. As residue, there are obtained 108.6 g. N-hydroxy-norpseudopelletierin. The compound is recrsytallised from ethyl acetate; m.p. 137—139°C. The yield is 51% of theory.

II. General procedure for the preparation of 5-nitrofurfurylidene derivatives of homophthalimide of Formula 1b):

0.05 mol homophthalimide or N-methyl-homophthalimide are dissolved in 100 ml. acetic anhydride. 7 g. (0.05 mol) 5-nitrofurfural are added thereto and the reaction mixture heated to 70°C. After some time, the precipitated material is filtered off with suction, washed with ether and recrystallised. The compounds set out in the following Table were prepared in this manner:

TABLE

Compound no.	R_2	period of reaction	yield	m.p. °C.	recrystallised from
XII	H	5 hours	80%	278	acetic acid
XIII	CH_3	1.5 hours	41%	226—227	dioxan

III. 1 - phenyl - 3 - methyl - 4 - (5 - nitro - 2 - furfurylidene) - pyrazol - 5 - one of Formula 1e) (compound no. XIV):

17.4 g. (0.1 mol) 1-phenyl-3-methyl-5-pyrazol-5-one and 14.1 g. (0.1 mol) 5-nitrofurfural in 150 ml. acetic anhydride are stirred for 3 hours at 100°C. After cooling, the reaction mixture is filtered with suction to give 28.7 g. of crude product. After recrystallisation from dioxan, there is obtained 1-phenyl-3-methyl-4-(5-nitro-2-furfurylidene)-pyrazol-5-one in a yield of 55.1% of theory. This compound melts at 234°C., with decomposition.

IV. 3 - (5 - nitro - 2 thienyl) - 2 - nitro - propane - 1,3 - diol and 2,2 - dimethyl-4 - (5 - nitro - 2 - thienyl) - 5 - nitro - 1,3 - dioxan of Formula 1c (compounds nos. XV and XVI):

6.28 g. 5-nitro-thiophene-2-aldehyde are dissolved in 40 ml. chloroform, 4.4 g. nitro-ethanol, dissolved in 20 ml. chloroform, are added thereto, the reaction mixture mixed with 0.4—0.6 ml. triethylamine and then left to stand at room temperature. After only a short time, an oil precipitates out which, after cooling and triturating, crystallises. After standing for several hours in a refrigerator, the product is filtered off with suction, washed with chloroform and there are thus obtained 9.1 g. (91.8% of theory) of a crude product with a melting point of 111—114°C. After recrystallisation from isopropanol-water (1 : 1), with the addition of a trace of *p*-toluene-sulphonic acid, there is obtained analytically-pure 3-(5-nitro-2-thienyl)-2-nitro-propane-1,3-diol with a melting point of 136—140°C.

1.24 g. of the crude diol obtained as above (m.p. 111—114°C.) are dissolved in 6 ml. anhydrous acetone, 12 ml. anhydrous benzene, as well as an intimate mixture of 0.6 g. phosphorus pentoxide and 0.6 g. kieselguhr, are added thereto, the reaction mixture is heated to 40—45°C., after 15 minutes a further 12 ml. benzene are added thereto and the reaction mixture thereafter stirred for about 6 hours at 40—45°C. Undissolved material is now filtered off with suction, washed with ethylene chloride into the filtrate, the filtrate neutralised with an aqueous slurry of calcium carbonate and undissolved material filtered off with suction. The aqueous phase of the filtrate is extracted with ethylene chloride. The organic phases are combined, dried over anhydrous sodium sulphate and evaporated in a vacuum. 1.4 g. of an oil thereby remaining behind. This is first triturated with ligroin and then with isopropyl ether, the crystals thus obtained filtered off with suction and then washed with isopropyl

ether. There are thus obtained 0.8 g. of crystals (55.5% of theory) with a melting point of 105—108°C. After recrystallisation from isopropanol, the melting point of the 2,2-dimethyl-4-(5-nitro-2-thienyl)-5-nitro-1,3-dioxan obtained increases to 107—109°C. (decomp.). The yield is 0.55 g.

V. 1 - (5 - nitro - 1 - methyl - 2 - pyrryl) - 2 - (4 - quinolyl) - ethylene of Formula 1d) (compound no. XVII):

1.68 g. 4-methyl-quinoline are heated for 4 hours under reflux (160°C.; bath temperature) with 1.85 g. 5-nitro-1-methyl-pyrrole-2-aldehyde in 12 ml. acetic anhydride. After cooling the reaction mixture, the precipitated yellow crystals are filtered off with suction, washed with a little acetic anhydride and then with isopropyl ether. There are thus obtained 0.6 g. (18% of theory) of paper chromatographically pure material which, after recrystallisation from 17 ml. dioxan, with the addition of activated charcoal, melts at 240—242°C. (decomp.). The yield is 0.45 g.

The following Examples are given for the purpose of illustrating the process according to the present invention and for illustrating some typical forms of the diagnostic agents according to the present invention: —

EXAMPLE 1.

General procedure for carrying out the detection of bacteria.

25.6 g. of one of the compounds of general formula (1) are dissolved in 5 ml. dimethyl formamide and 5 ml. polyoxyethylene sorbitan laurate ("Tween 20" ("Tween" is a Registered Trade Mark). With sterilised distilled water there is prepared a geometric dilution series (factor 0.5) which is diluted with a convention nutrient broth, such as that known under the Registered Trade Mark "Difco," in a ratio of 1:10. Accordingly, the individual solutions contain 256, 128, 64, 32, 16 etc. μg./ml. of the substance. Sterilised test tubes are each filled with 2 ml. of these solutions and mixed with the bacteria-containing sample to be tested. The test tubes are closed with a sterilised cottonwool swab and incubated at 21—37°C.: instead of an incubation cabinet, there can also be used a thermostatically-controlled water bath. In dependence upon the type of microorganism, the period of incubation and the number of microorganisms initially introduced, the test sample begins to become coloured. The colour and the time of coloration are compared with standard values and, in this manner, indicate the nature and number of the bacteria. Because of the bacteriostatic action of some of the compounds of general formula (1) to certain strains of microorganisms, only rather weak colorations or no colorations at all occur in the test samples with high concentrations of a compound of general formula (1). In general, therefore, only the test samples with the lower concentrations are evaluated.

In the following Tables I, II and III, there are summarised the typical colorations depending upon compound of general formula (1) used, the nature of the microorganism, the temperature and the incubation period. In general, the number of microorganisms is above 100,000/ml., i.e. the microorganism number present in cases of significant bacterurias. Microorganisms numbers of less than 100,000/ml. down to 10,000/ml. are found in cases of asymptomatic bacterurias and microorganism numbers below 10,000/ml. are regarded as being "normal".

TABLE I

Compound	incubation temp. in °C.	Escherichia coli		Streptococcus faecalis	
		coloration begins after hrs.	colour	coloration begins after hrs.	colour
I	37 21	5 12	red red-violet	2 12	green green
II	37 21	4 12	violet violet	1 12	green green
III	37	3	violet	1	yellow-green
IV	37	4	violet	1	green
V	37	18	brown	18	orange
VI	37	4	red-brown	1	brown
VII	37	4	red	1	green
VIII	37	5	dark violet	12	red
IX	37 21	5 12	brown brown	2 4	brown brown
X	37	18	red	—	—
XII	37	7	yellowish	7	green
XIII	37 21	3 10	red-brown greenish	2 5	green green
XIV	37	18	violet	18	violet
XVI	37	4	orange	4	yellow-green
XVII	37 21	4 12	green green	4 12	green green

TABLE II

Compound	incubation temp in . °C.	Proteus mirabilis		Staphylococcus aureus	
		colouration begins after hrs.	colour	colouration begins after hrs.	colour
I	37 21	4 12	brown brown	12 12	yellow-green yellow-green
II	37 21	4 12	violet violet	12 12	green green
III	37	4	red-brown	12	brown-red
IV	37	4	brown	12	yellow-green
V	37	18	orange	—	—
VI	37	5	red-brown	8	violet
VII	37	4	brown	12	yellow
VIII	37	5	brown	—	—
IX	37 21	4 12	brown brown	12 12	brown yellow-brown
X	37	18	red	—	—
XII	37	6	green	12	yellow-green
XIII	37 21	3 10	red red-brown	10 10	olive green greenish
XIV	37	18	violet	18	violet
XVI	37	12	yellow-orange	12	orange
XVII	37 21	7 12	greenish yellow-green	8 —	green —

TABLE III

Compound	Incubation temp. in °C.	Klebsiella pneumoniae		Pseudomonas aeruginosa	
		colouration begins after hrs.	colour	colouration begins after hrs.	colour
I	37 21	6 14	violet-brown brown	12 14	greenish greenish
II	37 21	4 12	violet violet	12 12	red-brown green-brown
III	37	4	violet	12	brown
IV	37	4	violet	12	greenish-brown
V	37	—	—	—	—
VI	37	12	red	12	red-brown
VII	37	5	red-brown	12	brown
VIII	37	12	brown	12	yellow
IX	37 21	12 12	red-brown yellow-brown	12 12	brown yellow-orange
X	37	18	brown	—	—
XII	37	7	orange-red	12	yellow-green
XIII	37 21	3 10	brown-green yellow-green	3 10	green green
XIV	37	18	violet	18	violet
XVI	37	6	orange	—	—
XVII	37 21	4 12	green green	12 —	yellow-green —

EXAMPLE 2.

Semi-quantitative determination of numbers of microorganisms.

a) *Escherichia coli*

There is used compound XVII at a concentration of 32 µg./ml and an incubation temperature of 37°C. When the number of microorganisms is 1000 to 10,000/ml., no colour change takes place even after 10 hours. In the case of numbers of microorganisms of 10,000 to 50,000/ml., the yellowish solution takes on a clear green colour after 10 hours. When the number of microorganisms is 50,000 to 100,000/ml., this coloration occurs after only 9 hours, when the number is 100,000 to 500,000/ml., after 8 hours and when the number is more than 500,000/ml., after only 7 hours.

b) *Klebsiella pneumoniae*

There is used compound II at a concentration of 64 µg./ml and an incubation temperature of 37°C. When the number of microorganisms is from 1000 to 100,000/ml., after 9 hours the yellowish solution becomes red-brown and after 10 hours violet. In the case of numbers of microorganisms of 100,000 to more than 500,000/ml., the red-brown colour appears after only 7—8 hours and the violet colour after only 9 hours.

c) *Proteus mirabilis*

There is used compound XIII at a concentration of 32 µg./ml. and an incubation temperature of 37°C. In the case of numbers of microorganisms of 4,000,000/ml. the pale yellow solution becomes red after only 4 hours. In the case of numbers of microorganisms of 400,000/ml., the colour change occurs after 6 hours, with 40,000/ml. after 7 hours, with 4000/ml. after 8 hours and with 400/ml. after 9 hours.

EXAMPLE 3.

1.8 ml. of nutrient broth containing microorganisms are incubated for 5 hours at 37°C. Thereafter, 0.2 ml. of a solution containing compound II at a concentration of 500 µg./ml. is added. (For the preparation of this solution, 10 mg. of compound II are dissolved in 2 ml. dimethyl formamide and 2 ml. polyoxyethylene sorbitan laurate ("Tween" 20), subsequently mixed with 6 ml. distilled water and then again diluted with distilled water in a ratio of 1:1). After incubating for half an hour, the coloration is evaluated. In the following Table, there are summarised the results of experiments with various microorganisms and various numbers of microorganisms.

TABLE IV

test organism	number of microorganisms/ml.		
	50,000—100,000	100,000—500,000	more than 500,000
Escherichia coli	—	weak violet	violet
Streptococcus faecalis	—	violet	violet
Proteus mirabilis	—	violet	violet
Staphylococcus aureus	—	—	violet-brown
Klebsiella pneumoniae	—	—	violet

As can be seen from this Table, substantially clearer colour changes are observed in the case of the numbers of microorganisms which are of significance in practice.

EXAMPLE 4.

In a manner analogous to that described in Example 3, nutrient broth containing microorganisms or a urine sample containing microorganisms is incubated for 5 hours

at 37°C., then mixed with 0.2 ml. of a solution of compound XVII, incubated for a further half an hour and subsequently evaluated. As in Example 3, the solution of compound XVII is prepared from 10 mg. of the compound in 2 ml. dimethyl formamide, 2 ml. polyoxyethylene sorbitan laurate and 6 ml. distilled water and subsequent dilution with distilled water in a ratio of 1:1. The results obtained with various types of microorganisms and various numbers of microorganisms are summarised in the following Table V.

TABLE V

test organism	number of microorganisms/ml.			
	10,000—50,000	50,000—100,000	100,000—500,000	more than 500,000
Escherichia coli	—	—	—	pale green
Streptococcus faecalis	—	—	green	green
Proteus mirabilis	—	—	green	green
Klebsiella pneumoniae	—	pale green	pale green	pale green

As can be seen from this Table, sharp limits of the colour change are observed in the case of the numbers of microorganisms which are of significance in practice.

EXAMPLE 5.

In 1.8 ml. of a urine sample containing microorganisms, there are dissolved the contents of a gelatine capsule which consist of 10 mg. soya peptone, 5 mg. dextrose, 10 mg. sodium chloride and 5 mg. dipotassium hydrogen phosphate, as well as the following additives which promote the reaction: 4 mg. NAD, 1 mg. cysteine and 0.1 mg. manganous chloride tetrahydrate.

The sample is incubated for 2 hours at 37°C. and then mixed with 0.2 ml. of a solution of compound II at a concentration of 500 μg./ml. (For the preparation of this solution, 10 mg. of compound II are dissolved in 2 ml. dimethyl formamide and 2 ml. polyoxyethylene-sorbitan laurate ("Tween 20"), subsequently mixed with 6 ml. distilled water and thereafter diluted again with distilled water in a ratio of 1:1). After an incubation time of half an hour, the coloration is evaluated for the first time. Further evaluations are carried out after further incubation for 1 and 2 hours.

In the following Table VI, there are summarised some typical results of this experimental method with numbers of microorganisms of about 3,000,000/ml.

TABLE VI

test organism	colour after further incubation		
	1/2 hour	1 hour	2 hours
Escherichia coli	violet	deep violet	deep violet
Proteus mirabilis	yellow	violet	violet
Klebsiella pneumoniae	pale violet	violet	violet
Streptococcus faecalis	violet	violet	violet
Staphylococcus aureus	yellow	pale violet	violet

In the case of parallel experiments with the same nutrient medium but without the additives which promote the reaction, after such short preliminary incubation times, colour changes which are visible to the naked eye cannot be ascertained.

EXAMPLE 6.

The colour which is formed in the case of the incubation of microorganism-containing solutions with compound II, can be measured in a photometer at 578 mμ even in the case of quite low concentrations. The following measurements, which were carried out on an aqueous suspension containing 30 million microorganisms per millilitre with the immediate addition of compound II, shew that, by the addition of 1 mg. NAD or NADP, the development of the colour commences substantially earlier. It is to be noted that at an extinction of about E=0.200, a change from yellow to violet can be clearly observed by the naked eye. The results obtained are set out in the following Table VII.

TABLE VII

incubation time	NAD	NADP	without addition
initial value	E = 0.150 (yellow)	E = 0.160 (yellow)	E = 0.102 (yellow)
1 hour	E = 0.180 (yellow)	E = 0.180 (yellow)	E = 0.130 (yellow)
2 hours	E = 0.475 (violet)	E = 0.400 (violet)	E = 0.200 (yellow)
3 hours	E = 0.710 (violet)	E = 0.680 (violet)	E = 0.445 (violet)
4 hours	E = 0.750 (violet)	E = 0.750 (violet)	E = 0.725 (violet)
5 hours	E = 0.680 (violet)	E = 0.680 (violet)	E = 0.700 (violet)

EXAMPLE 7.

In the same manner as described in Example 6, bacterial suspensions of *Escherichia coli* containing 630,000 microorganisms per millilitre and increasing amounts of NAD were incubated and evaluated photometrically. The extinction results (E) obtained are set out in Table VIII.

TABLE VIII

NAD concentration per reaction sample	2 hours	3 hours	4 hours	5 hours
0 mg.	0.079	0.071	0.098	0.120
1 mg.	0.196	0.690	0.745	0.638
2 mg.	0.234	0.720	0.745	0.630
3 mg.	0.274	0.760	0.750	0.690
4 mg.	0.304	0.820	0.760	0.650

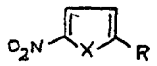
EXAMPLE 8.

Urine samples from clinical patients in which microorganism numbers of between 10,000 and 3,000,000 per millilitre had been determined in the usual manner, without

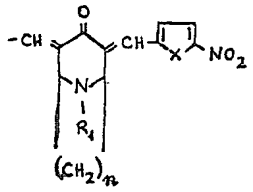
a specification of the nature of the microorganisms being known, were investigated in the manner described in Example 5. After a preliminary incubation of only 2 hours and a further incubation of half an hour, the urine samples shewed a violet colour, indicating more than 2,000,000 microorganisms per millilitre. Urine samples containing about 100,000 microorganisms per millilitre became violet coloured after a further incubation period of 1—2 hours.

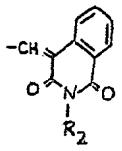
WHAT WE CLAIM IS:—

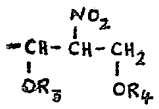
1. A diagnostic agent for the detection of bacteria, comprising a liquid or solid nutrient medium and a non-growth-inhibiting amount of a compound of the general formula:—

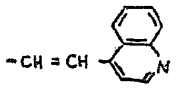


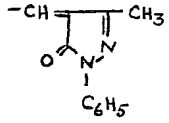
in which X is an oxygen or sulphur atom or an alkylated nitrogen atom and R is a radical of the general formula:—











in which R_1 is a saturated or unsaturated aliphatic, aromatic, araliphatic, acyl, hydroxy or acyloxy-aliphatic radical, R_2 is a hydrogen atom or lower alkyl radical, R_3 and R_4 are both hydrogen atoms or together represent an isopropylidene radical and n is 2 or 3.

2. A diagnostic agent according to claim 1, wherein there is additionally present a solubiliser for the compound.

3. A diagnostic agent according to claim 1 or 2, wherein there is additionally present at least one co-enzyme.

4. A diagnostic agent according to claim 3, wherein the coenzyme is nicotinamide-adenine-dinucleotide, nicotinamide-adenine-dinucleotide phosphate or a reduction product thereof.

5. A diagnostic agent according to any of the preceding claims, wherein there is additionally present a sulphhydryl group-containing compound or a corresponding dehydrated disulphide compound.

6. A diagnostic agent according to claim 5, wherein the sulphhydryl group-containing compound is cysteine, reduced glutathione, cysteamine or β-mercapto-ethanol.

7. A diagnostic agent according to any of the preceding claims, wherein enzyme-activating heavy metal ions are additionally present.

8. A diagnostic agent according to claim 7, wherein the enzyme-activating heavy metal ions are manganese or copper ions.

9. A diagnostic agent according to any of the preceding claims, whenever in the form of a tablet or impregnated filter paper.

10. A diagnostic agent according to any of claims 1 to 8, wherein the compound of the general formula given in claim 1 is in the form of a stable solution and the other components are in a gelatine capsule, from which they can be removed and dissolved in water prior to use.

11. Diagnostic agents according to claim 1, substantially as hereinbefore described and exemplified.

12. Process for the detection of bacteria, wherein the material to be investigated is brought together with a solid or liquid nutrient medium and a non-growth-inhibiting amount of a compound of the general formula given in claim 1, whereafter the colour change is evaluated.

13. Process according to claim 12, wherein the colour change is evaluated after incubation for 1—18 hours at 20—37°C.

14. Modification of the process according to claim 12, wherein the material to be investigated and the solid or liquid nutrient medium are first incubated, a compound of the general formula given in claim 1 then added and incubation continued, whereafter the colour change is evaluated.

15. Process according to any of claims 12 to 14, wherein there is additionally used at least one coenzyme and/or sulphhydryl group-containing compound and/or enzyme-activating heavy metal ions.

16. Process according to any of claims 12 to 15, whenever applied to urine, milk or drinking water.

17. Process according to any of claims 12 to 16 for the detection of bacteria, substantially as hereinbefore described and exemplified.

18. The use of compounds of the general formula given in claim 1 for the detection of bacteria.

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